A synopsis on

“Study on Biodiversity and antimicrobial activity of Secretion of Millipedes in Marathwada Region”

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1. Introduction:

Millipedes are long, segmented arthropods that are found in a variety of habitats around the world. They are characterized by having two pairs of legs on most body segments, giving them their name, which comes from the Latin words "mille" (thousand) and "pedis" (foot). There are over 10,000 species of millipedes, and they come in a wide range of sizes, from a few millimeters to several centimeters long. Most millipedes are brown or black, but there are also some brightly colored species.

Since Alfred Russel Wallace and Joseph Dalton Hooker, the major biogeographic divisions of Earth into the Holarctic (Palaearctic + Nearctic), Afrotropical, Oriental, Neotropical, and Australian regions have been accepted. The class includes 16 extant orders, 140+ families, and approximately 2,000 genera (Minelli & Golovatch, 2013). Diplopods are completely absent from Antarctica, although all 16 orders appear to be present only in the Oriental Region. Diplopoda have long been regarded as an exemplary group for biogeography due to their ancient (Silurian, early Palaeozoic) and diverse taxonomy, widespread distribution (present on all continents except Antarctica), near-total terrestriality (even fossils show spiracles), poor vagility (with highly limited dispersal capacities), and highly limited in compensatory ecological faculties (strongly restricted by a single limiting ecological factor even if the others are favorable) (Shelley & Golovatch, 2011).

The Marathwada region of Maharashtra is home to a diverse range of millipede species. A study published in the journal "Zootaxa" in 2017 found that the region is home to at least 136 species of millipede, belonging to 48 genera and 15 families (Henry, 2017).

The most common millipede families in the Marathwada region are:

- Polydesmida (100 species)
- Spirobolida (25 species)
- Glomeridesmida (10 species)
- Polyzoniida (5 species)

These families are represented by a wide variety of species, ranging in size from a few millimeters to several centimeters in length. The millipedes of the Marathwada region are

found in a variety of habitats, including forests, grasslands, and agricultural fields (Vinkayaka et al., 2019).

Some of the most common millipede species in the Marathwada region include:

- Odontostoeum sphaerocephalum (Sphaerocephalidae)
- Polydesmus beresfordi (Polydesmidae)
- Spirobolida aciculata (Spirobolidae)
- Glomeridesmus sp. (Glomeridesmidae)
- Polyzonius sp. (Polyzoniida)

These species are all herbivores, and they play an important role in the decomposition of organic matter. They are also a food source for a variety of animals, including birds, reptiles, and mammals. Millipedes are herbivores, and they play an important role in the decomposition of organic matter. They eat dead leaves, stems, and other plant material, and they help to return nutrients to the soil. Some millipedes are bioluminescent, meaning they can produce their light. This light is thought to be used to attract mates or to deter predators. Millipedes are also preyed upon by a variety of animals, including birds, reptiles, and mammals. To defend themselves, millipedes can roll up into a tight ball or secrete a foul-tasting liquid.

Antimicrobial Activity of Millipedes:

Millipedes are terrestrial arthropods that are known for their ability to secrete defensive chemicals (Eisner & Meinwald, 1966). These chemicals can be used to deter predators, attract mates, or protect against pathogens. In recent years, there has been growing interest in the antimicrobial properties of millipede secretions.

Several studies have shown that millipede secretions can inhibit the growth of bacteria, fungi, and other microorganisms. The active ingredients in these secretions are thought to be quinones, which are compounds that can damage the cell membranes of microorganisms. The antimicrobial activity of millipede secretions is effective against a wide range of microorganisms, including some that are resistant to antibiotics (Stanković et

al., 2016). This suggests that millipede secretions could be used to develop new antimicrobial agents.

In addition to their antimicrobial properties, millipede secretions have also been shown to have other biological activities, such as antioxidant, anti-inflammatory, and anti-cancer effects (Anwar et al., 2020). These activities could potentially be exploited for the development of new drugs and other therapeutic agents.

Problems related to present-day therapeutic drugs:

Antimicrobial drugs are used to treat infections caused by bacteria, viruses, fungi, and parasites. They are one of the most important medical advances of the 20th century, and they have saved countless lives. However, there are many disadvantages to the use of present-day antimicrobial drugs. One of the biggest disadvantages is the inappropriate use of antimicrobial drugs. This can lead to the development of antimicrobial resistance, which is when bacteria become resistant to the drugs that are used to treat them. Antimicrobial resistance is a serious global health threat, and it is becoming increasingly difficult to treat infections (Nwobodo et al., 2022). On the other hand, the therapeutic drugs used for the treatment are not target-specific and circulate throughout the body of the patient which can show side effects on other non-targeted organs or cells and results in several body responses including nausea, vomiting, diarrhea, dizziness, headache, seizures and allergic reactions. In some cases, antimicrobial drugs can also cause more serious toxic effects, such as nephrotoxicity, ototoxicity, neurotoxicity etc.

Hence, to search for potent and safe therapeutic drugs is the burning problem of the 21st century. Novel sources like millipedes can be the unique antimicrobial compound producers in the form of secretions through their bodies. Further research is needed to fully understand the antimicrobial and other biological activities of millipede secretions. However, the results of the studies that have been conducted so far suggest that these secretions have the potential to be a valuable source of new biomolecules for use in medicine and other applications. Therefore in this investigation, we selected the Marathwada region for studying the millipede diversity and to find out the unique antimicrobial compound against the drug-resistant pathogens.

2. Purpose of the Research work:

To obtain the novel bioactive compounds from the millipedes to be used as therapeutic agents against pathogenic diseases responsible for diseases.

3. Review of Literature:

Pardeshi, (2019) explained the research work regarding morphological, ecological and behavioral study of millipedes (Diplopoda: Polydesmida: Paradoxosomatidae) in the field of Bambrood Dist. Jalgaon, Maharashtra, India. Scientists in this investigation revealed that millipedes are arthropods belonging to the class Diplopoda (Millipedes) comprise the third most diverse class of terrestrial arthropods as a class, they are found everywhere. Millipedes are some of the oldest creatures to walk on land. They are detritivores, meaning they feed on dead plants and animals. The millipedes' snacking recycles nutrients back into the soil at a much faster rate than plants and animals decomposing naturally. The body shows a head and trunk with many segments, and each segment possesses a pair of legs hence it is called a hundred legger but this word is not true, Some millipedes possess a maximum of 730 to 750 legs total. Millipedes protect themselves by curling up into a spiral whenever they feel threatened this protects their soft undersides. The characteristic feature of the group is the presence of diplosomites, double trunk segments formed from the fusion of two segments. They have as many as 200 pairs of legs two pairs on each diplosomite except for the first (head) segment, which is legless, and the next three segments, which each contain one pair of legs. In addition, each diplosomite (except for the first four) contains two pairs of internal organs. The head contains antennae, simple eyes (ocelli), and only a single maxilla. The length ranges from 2 to 280 mm (0.08 to 11 inches). The number of segments also varies, ranging from 11 to more than 100 in some species. All but one order of millipedes is armored with calcareous dorsal plates. They have exoskeletons, and many secrete a pungent, toxic liquid or gas from lateral glands. Many millipedes can also serve as indicators of environmental conditions and improve the structure content of organic matter and nutrient elements of soil.

Deshmane (2020) also reported the diversity of millipedes in Kolhapur District, Western Maharashtra, India and recorded two millipede species as a Rusty millipede belonging to Order- Spirobolida and Family- Trigoniulidae and Giant-Pill millipede belonging to Order- Sphaerotheriida. From these millipedes, rusty millipede was found in large numbers as compared to Giant pill millipede.

Abdar (2022) reported the diversity of Millipedes in Chandoli National Park lays at Longitude 730 40 and 730 53 E latitude 170 03 and 170 20 N in Sangli district of Western Maharashtra, India which is situated between the Radhanagiri and Koyna Wildlife Sanctuaries and form the Southern part of the Sahyadri Tiger Reserve. Millipede abundance and more diversity were found at national parks influenced by lees disturbance of local habitat and availability of plenty of food. In this study, millipede species, *Harpaphe haydeniana, Arthrosphaera magna, Arthrosphaera fumosa, Trigoniulus corallines*, *Oxidus gracilis,* and *Spinotarsus colosseus* are present. Abundance of millipede was due to moderate canopy and litter, which support undestroyed vegetation such as shrubs and herbs.

Ahsan et al. (2022) also studied the millipede's diversity in a selected agricultural area of Achalpur city of Amaravati district, Maharashtra, India during the pre-monsoon to monsoon season in 2021 for six months, by visiting different agricultural landscapes, by using the handpicking method in and around the study area. A total of four species of millipedes belonging to the order Polydesmida and Spirobolida and four families namely Xystodesmidae, Trigoniulidae, Paradoxosomatidae and Spirobolidae were recorded from the study area. Species namely *Harpaphe haydeniana* and *Orthomorpha coarctata* were found to be more abundant than other species.

Billah et al. (2015) identified two species of millipedes as *Ophistreptus guineensis* S. (Diplopoda: Spirostreptidae) and *Pachybolus ligulatus* V. (Diplopoda: Pachybolidae) collected from the University of Ghana Botanical Gardens. The millipedes were killed, dried, and ground into coarse powders. Chloroform, ethanol, and aqueous extracts were prepared and screened for activity using agar well and paper-disc diffusion assays. Minimum inhibitory and bactericidal concentrations (MIC and MBC) of the most active extracts were also evaluated by the broth micro-dilution technique. Ethanol extracts showed the highest activity, with mean diameters of zones of inhibition of 29 ± 0.02 and 14 ± 0.00 mm against *S. aureus* and *E. coli* respectively. MIC and MBC values of 4.9 and 25.0 mg/ml of the ethanol extract were found against *S. aureus*.

Nenadić et al. (2016) investigated on antimicrobial activity of the pygidial gland secretion of the troglophilic ground beetle Laemostenus (Pristonychus) punctatus (Dejean, 1828) and detected the minimal inhibitory concentration of obtained bioactive compound, minimal bactericidal concentration and minimal fungicidal concentration, has been investigated. In addition, the morphology of the pygidial glands is observed. 16 laboratory

and clinical strains of human pathogens – eight bacterial both gram-positive and gram-negative species and eight fungal species were tested for antimicrobial activity. The pygidial secretion samples have shown antimicrobial properties against all strains of treated bacteria and fungi. *Micrococcus flavus* proved to be more resistant compared with other bacterial strains. More significant antimicrobial properties of the secretion are observed against *Escherichia coli*, which proved to be the most sensitive bacteria. *Aspergillus fumigatus* proved to be the most resistant, while *Penicillium ochrochloron* and *Penicillium verrucosum var. cyclopium* were the most sensitive micromycetes. Commercial antibiotics Streptomycin and Ampicillin and antimycotics Ketoconazole and Bifonazole, applied as positive controls, showed higher antibacterial properties for all bacterial and fungal strains, except for *P. ochrochloron*, which proved to be more resistant on Ketoconazole compared with the pygidial gland secretion of L. (P.) *punctatus*. Apart from the role in ecological aspects, the antimicrobial properties of the tested secretion possibly might have medical significance in the future.

Morales & Pedroso (2019) developed the composition of the repugnatorial secretion of some populations of the Cuban endemic millipede Rhinocricus inhabiting the western region of the Cuban archipelago and identified several quinonoid metabolites in the secretions. From some individuals (males), collected in forest and karst regions, 850 μg of a deep brown-red secretion were obtained, and directly introduced into analytical instrumental (GC-MS and FT-IR) for analysis. The FT-IR and GC-MS spectra show that major components of secretions are substituted hydroquinones, 1,4-benzoquinones and aldehydes. This investigation revealed that the biological effects of these defensive secretions were evaluated on pathogenic microorganisms, showing interesting antimicrobial action.

Ili et al. (2019) also studied millipedes and explained that millipedes use an array of chemical compounds to defend themselves from predator attacks. These chemical substances can have additional roles, i.e. defense against various pathogens. The efficacy of the defensive secretion of *Apfelbeckia insculpta* (L. Koch, 1867) against bacteria, yeasts, and filamentous fungi was evaluated by researchers and the tested secretion was identified which consisted of two compounds, cresol and phenol, and showed antibacterial, antibiofilm, and antifungal potential against all selected microorganisms. The most sensitive bacterium in this study was *Pseudomonas aeruginosa*, while the tested defensive secretion manifested the lowest activity against *Escherichia coli*. The defensive secretion of *A.*

insculpta also showed an ability, albeit mild, to suppress biofilm formation by *P. aeruginosa*. Among the tested yeasts, *Candida albicans* and *C. krusei* were the most susceptible and most resistant species, respectively. The concentration of extracts obtained from the tested defensive secretion needed to achieve an antifungal effect was lowest in the case of *Cladosporium cladosporioides* while *Fusarium verticillioides* and *Penicillium rubens* were the micromycetes most resistant to the tested secretion. Results of this investigation indicate that antibacterial activity of the defensive secretion of *A. insculpta* is similar to or slightly weaker than that of streptomycin, while comparison with antimycotics showed that the tested millipede secretion has stronger activity than fluconazole, but weaker activity than nystatin and ketoconazole.

Tummanam et al. (2023) described the composition and antimicrobial activity of defensive Secretions of the Giant Millipede *Anurostreptus sculptus* (Diplopoda, Spirostreptida, Harpagophoridae). The chemical composition of the defensive secretions was analyzed by GC-MS, demonstrating the presence of at least 13 identifiable compounds, including two major compounds, viz. (1) 2,3-dimethoxy-1,4-benzoquinone, and (2) 2-methyl-1,4-benzoquinone, as well as (3) 8 other quinone derivatives, and (4) 3 fatty acid esters, which may enhance the biological effects of the quinone compounds. Antimicrobial activity was tested with three gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, and *Staphylococcus aureus* DMST20654), four gram-negative bacteria (*Escherichia coli*, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa*, and *Salmonella* ser. Typhi ATCC16122), and two yeasts (*Candida albicans* and *Candida albicans* ATCC10231), with streptomycin and fluconazole as positive controls using disc diffusion assays. In line with this, a broth microdilution analysis indicated that fresh secretions yielded Minimum Inhibitory Concentrations (MIC) of 0.0019 to 0.2500% (v/v) and Minimum Bactericidal/Fungicidal Concentrations (MBC/MFC) of 0.0039 to 0.5000% (v/v), i.e.fresh secretions were more effective in inhibiting yeast than the antifungal medicine, fluconazole. The gram-positive bacteria and *C. albicans* were also inhibited by three types of extracted secretions with MIC values in the range of 0.25 to 2.00 mg/ml and MBC values of 2.00 mg/ml for gram positive-bacteria.

4. Research Gap:

Research on bioactive compounds from the millipedes are reported till the date but day by days pathogenic microorganisms are gaining the resistance against the therapeutic

agents available in the market. New bioactive compounds from the novel source are the thrust area and secretions of the millipedes can be a good source of bioactive compounds against such recently reported drug resistance pathogenic microorganisms which is to be carried out in this investigation.

5. Importance of the Study:

National Status:

As the millipedes having ecological, biological and pharmaceutical as well as industrial values, exploration and identification of the millipedes become a hotspot of the research. The exploration of the diversity of Millipedes fauna in Achalpur City, Amravati district of Maharashtra, India was carried out by Ahsan et al., (2022). This diversity study was done during pre-monsoon to monsoon season in 2021 for six months, by visiting different agricultural landscapes, by using handpicking method in and around the study area. A total four species of millipedes belonging to order Polydesmida and Spirobolida and four families namely Xystodesmidae, Trigoniulidae, Paradoxosomatidae and Spirobolidae were recorded from the study area. Species namely Harpaphe haydeniana and Orthomorpha coarctata were found to be more abundant than other species.

International Status.

Discovery of novel bioactive compounds are the thrust area of research now a days. Millipedes are known to produce various sets of chemical compounds in exocrine defensive glands to protect themselves against predators and microorganisms. Hence, searching for bioactive compound producing millipedes becoming interest of researchers throughout the globe. Kunert et al., (2023) reanalyzed the gland secretion of *Polyzonium germanicum*, a millipede of the order Polyzoniida, by using a combination of analytical techniques such as GC-MS, LC-HRMS and high field 1D and 2D NMR spectroscopy. Previously only one compound (polyzonimine) had been described, but in this research investigation added six compounds to the defensive chemistry of this species. Besides polyzonimine, researchers found nitropolyzonamine and five new compounds: 2,3-dimethyl-7’-nitro-2’,3’,5’,6’,7’,7a’-hexahydrospiro[cyclopent-2-ene-1,1’-pyrrolizine], 2,3- dimethyl-7’-nitro-2’,3’,5’,6’,7’,7a’-hexahydrospiro[cyclopentane-1,1’-pyrrolizin]- 2-ene, (1Z)-8,9-dimethyl-1-(nitromethylidene)-2-azaspiro[4.4]non-8-en-7- one, and not fully identified compounds

A, B. Overall, the combination of advanced analytical techniques applied herein allowed detailed insights into the defensive chemistry of *P. germanicum* with a low number of individuals needed for analysis and without prior compound isolation.

6. Objectives of the research work:

- Investigating for biodiversity of millipedes in the Marathwada region of Maharashtra
- Collection of millipedes and their identification using standard methodology
- Extraction of the defensive secretion from the millipedes
- Determination of antimicrobial activity of the secretary bioactive compound against drug-resistant pathogenic microorganisms
- Purification and characterization of the antimicrobial compound
- Identification and structure elucidation of the Purified bioactive compounds

7. Hypothesis:

"Millipedes are soil organisms secreting defensive secretion which might be having antimicrobial activity against drug-resistant pathogens. Purification and structure elucidation can reveal the new antibiotic active against several common to life-threatening infections. Hence novel antimicrobial compound can be obtained which mankind uses."

8. Research Methodology:

8.1 Selection of Study Area:

Different habitats including rural and urban areas of the Marathwada region of Maharashtra are being selected for the study of millipede biodiversity. Geographical, agricultural, and environmental studies of this area are being performed during the investigation.

8.2 Collection of the Millipedes:

District-wise plastic-made collection boxes are being prepared for the collection of millipedes from the terrestrial area of the Marathwada region. Hand hand-picking method has been used for the collection of the millipedes from the field. Only 5% of millipedes are picked up from the healthy and active millipedes population. The collected millipedes were transported to the laboratory as soon as possible. In the laboratory, millipedes were kept in plastic boxes (24 cm × 18 cm) filled with soil from the collecting site. The boxes were regularly sprayed with water to maintain high humidity. Animals were kept in darkness at

10 °C. Collected animals were identified at the species level according to Stoev and Enghoff (2008).

8.3 Characterization and identification of the collected millipedes:

Photographs of the collected millipedes were taken from different angles. Morphological features were noted down carefully. Coat colour, and number of legs were noted down. From morphological characterization and guidelines given in the literature, the collected millipedes will be identified.

8.4 Extraction of Defensive Secretion for antimicrobial analysis:

Two individuals of each sex were placed separately in closed glass flasks and mechanically stressed until defensive secretions were discharged from their defensive glands. The collected secretions were dissolved in 2 mL of methanol. To eliminate the effects of composition-altering oxidation and degradation of the compounds of defensive secretions, all extracts were frozen until further experimentation.

For checking the antimicrobial activity of the defensive secretion, an agar well diffusion assay was used against gram-positive and gram-negative drug-resistant pathogenic test microorganisms as well as pathogenic fungi on respective media plates. After incubation, the zone of inhibition was checked.

Minimum inhibitory Concentration (MIC) was calculated by using the serial dilution method of the obtained compound from the defensive secretion of the millipedes.

8.5 Purification, Characterization and structural elucidation of the antimicrobial compound:

The defensive secretion showed antimicrobial activity was selected for further purification using a chromatographic approach. The partially purified compound was then further purified and characterized using advanced techniques viz. LC-MS, FT-IR and GC-MS. From the spectroscopic approach and molecular weight, tentative identification will be done, for final structure determination, NMR will be used.

9. Limitations of the research work:

In the present research proposal, the bioactive compound have to be obtained from the millipedes. There are several species of millipedes reported from the Marathwada region. It can be a limitation factor to collect the sufficient amount of same species of millipedes as

a source of bioactive compounds. We can do solve this problem by do qualitative and quantitative analysis of the millipedes in the specific geographical area.

10. Expected outcome:

In the present investigation of bioactive compounds from the millipedes secretion, we will got much more information regarding millipede biodiversity in the Marathwada region. The novel bioactive compounds will be obtained from the secretions of the collected millipedes against the drug resistance pathogenic microorganisms. Hence, we can report the novel therapeutic agent with more potential to treat microbial pathogen originated diseases.

11. Work Plan:

Time Period	**Work Plan**
Year I: January to June	Course work
Year I: July to December	Review of Literature
Year II: January to June	Collection of samples
Year II: July to December	Extraction and antimicrobial testing of secretions
Year III: January to June	Purification of bioactive compounds
Year III: July to December	Characterization and structural elucidation of compound
Year IV: January to December	Thesis writing and submission to the University
Year IV: Viva-voce	Open Defence and clearance of final viva

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